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Oomycete-specific ITS primers for identification and metabarcoding

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Abstract

Microbial metabarcoding studies using high throughput sequencing technologies generate unprecedented amounts of DNA sequence data and make it possible to determine not only the composition of the communities but also the underlying factors powering the evolution of these communities. Despite the potential of community level studies in helping to better understand the ecology of pathogens and to manage the losses caused by them, very few oomycete addressing metabarcoding studies have been carried out and with highly variable results. The aim of this study was to develop new oomycete-specific ITS region PCR primers with improved specificity for metabarcoding and identification of oomycetes. The modified ITS1oo and the newly developed ITS3oo primers show improved *in silico* specificity for oomycetes and when paired with the universal ITS4 successfully amplified the DNA from all eleven tested oomycete species from six genera. High throughput sequencing of 20 soil samples from forest nurseries and bordering areas, using the primer pair ITS1oo/ITS4, recovered more than 400 oomycete OTUs, which is a significant increase over previous studies, and indicates the ability of the new method to detect various oomycete groups from complex substrates. The average fraction of oomycete reads per soil samples was 32–36%, with a maximum of 69%. The recovered oomycete OTUs represented the groups *Lagenidiales*, *Peronosporales*, *Pythiales* and *Saprolegniales*, with *Pythiales* dominating in all samples. In addition, the new primers were successfully used in identifying pathogens directly from infected plant tissues with Sanger sequencing. The pathogen was identified to the species or genus level in four samples out of six. In conclusion, the developed oomycete-specific primers provide a reliable method for the identification and metabarcoding of oomycetes.

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Key words
oomycete, community barcoding, next generation sequencing, ITS, community analysis, soil community

Introduction

Oomycetes are microscopic stramenopiles that are found in both aquatic and terrestrial environments (Sparrow 1960, 1976, Karling 1981, Dick 2001). Many oomycete species are important pathogens, causing serious economic losses by infecting vegetables, berries, trees, arthropods and vertebrate animals (Kamoun 2003, Herrero et al. 2011). Molecular methods enable rapid identification of pathogens in environmental samples and infected tissues by using specific PCR primers and rapidly evolving high-throughput sequencing (HTS) technologies. For oomycetes, the cytochrome c oxidase subunit 1 (cox1), the internal transcribed spacer (ITS) (Robideau et al. 2011, Vettraino et al. 2012) and the cytochrome c oxidase subunit 2 (cox2) (Choi et al. 2015) have been identified as suitable barcodes. The choice of metabarcoding primers that cover all known oomycete taxa and discriminate other groups, however, is still limited by the inconsistent performance of some existing oomycete-specific primers.

Robideau et al. (2011) evaluated the cox1, the ITS and the large ribosomal subunit (LSU) for use in DNA barcoding of oomycetes and suggested using cox1 and ITS in parallel due to their similar performance in resolving oomycete species and with both having superior performance in certain groups. Choi et al. (2015) compared the perfomance of cox1 and cox2 and found the latter to be more easily amplified across a wide range of oomycetes with existing primer sets. They also determined that the cox2 was more efficiently amplified from historic herbarium specimens and noted that in case of cox2 there is existing sequence data for several historic type specimens. As a result, Choi et al. (2015) suggested using the cox2 in addition to the ITS for oomycete barcoding. Additionally, Choi et al. (2015) proposed that for below species-level resolution the cox2-1 spacer could be used. For the cox2, there are also internal primers that can be used to amplify a 350 bp fragment suitable for barcoding (Hudspeth et al. 2000).

Of oomycete-specific ITS primers, ITS6 and ITS7 (Cooke et al. 2000) have been used for community studies, but with notable difficulties, as Coince et al. (2013) recovered only a small percentage of oomycete sequences using these primers. Sapkota and Nicolaisen (2015) optimized the ITS6/ITS7 assay by raising the annealing temperature and as a result improved the specificity of the primers. Other studies, however, have suggested that taxon recovery could be increased by using lower annealing temperatures (Ishii and Fukui 2001, Acinas et al. 2005) or multiple annealing temperatures (Schmidt et al. 2013). It is also advisable to re-optimize the PCR reaction whenever the reaction mixture is altered (Innis et al. 1990).

Another oomycete-specific ITS primer, the ITS-O, has been published by Bachofer (2004). Whereas this primer has so far not found use in oomycete community studies, it has been used successfully to amplify the DNA of a wide range of oomycetes in phylogenetic research (Spring et al. 2006, Thines 2007).

The aim of the current study was to develop new oomycete ITS primers with improved taxon coverage and specificity for use in community-level studies. In order to reduce material costs, we decided to develop two oomycete specific forward primers that can be combined with various universal reverse primers. The new and existing primers were analyzed *in silico* to evaluate the coverage and specificity of the primers and the primers selected as suitable for oomycete ITS barcoding were tested *in vitro* on cultures, infected plant tissues and soil samples.

Methods and materials

Pure cultures of oomycetes and fungi

DNA extracts from the pure cultures of eleven oomycete species from six genera were used in testing of the primers. Additionally, DNA from the cultures of five fungal species was used to test the specificity of the primers (Table 1).

Sampling and DNA extraction

A total of 20 soil samples were collected from beds of forest nurseries and bordering control areas (Table 2). Each sample consisted of 40 subsamples, which were taken

Table 1. Cultures of oomycetes and fungi that were used in testing the specificity of the new primers.

Species	Isolation year	Strain/culture code	Host
Achlya oligochanta	2010	HJ33C	*Astacus astacus*
Aphanomyces astaci	2008	KTY3-4	*Astacus astacus*
Aphanomyces astaci	2003	UEF8866-2	*Pacifastacus leniusculus*
Aphanomyces astaci	2014	AT1D	*Austropotamobius torrentium*
Aphanomyces laevis	2008	KTY5-2	*Astacus astacus*
Aphanomyces stellatus	2010	HJ38C	*Astacus astacus*
Aphanomyces salsuginosus	2014	NJM0912	*Salangichithys microdon*
Phytophthora infestans	2014	An2-13	n/a
Phytophthora infestans	2014	HiPa1-13	n/a
Phytophthora infestans	2014	Ti17-13	n/a
Phytophthora cactorum	2012	145714	*Betula pendula*
Pythium sp.	2007	T4B	*Astacus astacus*
Saprolegnia australis	2007	S23	*Astacus astacus*
Saprolegnia parasitica	2007	S14	*Astacus astacus*
Scoliolegnia sp.	2007	S16	*Astacus astacus*
Alternaria infectoria	2014	TU-3 TFC 2013-46	n/a
Armillaria cepistipes	2013	EPS 110	*Fraxinus excelsior*
Fusarium culmorum	2014	KV-6 TFC 2013-54	n/a
Neonectria radicicola	2013	EPS 82	n/a
Ulocladium castanea	2014	CBS 124390	n/a

Table 2. Soil samples used in evaluating the performance of the new oomycete specific primer pair ITS1oo/ITS4 in high-throughput sequencing.

Sample	Geocode	Time of sampling	Sampling plot description
1.1	58°48.00'N, 24°30.03'E	10/16/2014	1 y/o *Picea abies* nursery bed
1.2	58°48.02'N, 24°30.00'E	10/16/2014	Area bordering the nursery
2.1	58°20.51'N, 24°36.58'E	17/10/2014	Former *Picea abies* nursery bed
2.2	58°20.53'N, 24°36.42'E	17/10/2014	Area bordering the former nursery
3.1	59°9.49'N, 26°16.94'E	14/10/2014	2 y/o *Betula pendula* nursery bed
3.2	59°9.48'N, 26°16.67'E	14/10/2014	Area bordering the nursery
4.1	59°20.14'N, 26°51.37'E	9/26/2014	1 y/o *Picea abies* nursery bed
4.2	59°20.14'N, 26°51.36'E	9/26/2014	Area bordering the nursery
5.1	59°33.77'N, 26°5.60'E	9/25/2014	3 y/o *Picea abies* nursery bed
5.2	59°33.76'N, 26°5.60'E	9/25/2014	Area bordering the nursery
6.1	59°32.29'N, 26°16.40'E	9/25/2014	1 y/o *Picea abies* nursery bed
6.2	59°32.29'N, 26°16.36'E	9/25/2014	Area bordering the nursery
7.1	59°12.08'N, 26°13.67'E	11/5/2014	1 y/o *Betula pendula* nursery bed
7.2	59°11.89'N, 26°14.40'E	11/5/2014	Area bordering the nursery
8.1	56°6.22'N, 27°17.89'E	11/6/2014	1 y/o *Picea abies* greenhouse nursery bed
8.2	56°6.16'N, 27°17.98'E	11/6/2014	Area bordering the nursery
9.1	58°2.18'N, 25°58.17'E	11/7/2014	2 y/o *Picea abies* nursery bed
9.2	58°2.08'N, 25°57.97' E	11/7/2014	Area bordering the nursery
10.1	58°10.46'N, 26°11.18'E	11/7/2014	1 y/o *Betula pendula* nursery bed
10.2	58°10.44'N, 26°11.16'E	11/7/2014	Area bordering the nursery

Table 3. Symptomatic plant samples with oomycete infection that were used in testing of the new primers ability to identify pathogens directly from infected plant tissues.

Plant sample	Sampling location	Time of sampling	Host species / symptoms	Identified pathogen / sequence similarity
1.	Tartu county	06/11/2014	*Alnus incana*/ bark discolouration	*Phytophthora* sp./ 99%
2.	Tartu county	07/09/2014	*Solanum tuberosum*/ leaf rot	*Phytophthora infestans*/ 100%
3.	Tartu county	07/17/2014	*Aegopodium podagraria*/ leaf discolouration	*Plasmopara nivea*/ 99%
4.	Tartu county	09/14/2014	*Solanum lycopersicum*/ leaf rot	*Phytophthora infestans*/ 99%
5.	Tartu county	09/14/2014	*Vitis vinifera*/ leaf discolouration	No result
6.	Tartu county	n/a	*Cucurbita pepo*/ leaf discolouration	No result

with a 5 cm diameter sterile plastic pipe from the top 5 cm soil layer of a 50x50 m plot. The subsamples were pooled, dried and thoroughly mixed following Tedersoo et al. (2014). In addition, six samples of plant tissues with signs of oomycete infection were collected by excising a part of the symptomatic tissues (Table 3).

DNA was isolated from 2 g of soil with the MO BIO PowerMax Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). DNA from symptomatic

plant samples was isolated with the MO BIO PowerSoil DNA Isolation Kit from up to 0.2 g of material. Prior to DNA extraction, soil and tissue samples were crushed using bead beating with two 3.2 mm diameter stainless steel balls (BioSpec Products, Bartlesville, OK, USA). The 2 ml tubes containing the samples and balls were shaken for 5 min at 30 Hz with the Retsch Mixer Mill MM400 (Retsch, Haan, Germany). Pure culture DNA was extracted using a proteinase K-based method (100 µl 0.2 M $(NH_4)_2SO_4$ and 2.5 µl proteinase K; incubation at 56 °C for 24 h and at 98 °C for 15 min).

Primer design

The new oomycete-specific forward primers ITS1oo and ITS3oo were selected by aligning all oomycete and other stramenopile ITS sequences present in the International Nucleotide Sequence Databases (www.insdc.org). ITS sequences were aligned with MAFFT (http://mafft.cbrc.jp/alignment/software/) and checked by using SEAVIEW software (http://doua.prabi.fr/software/seaview). Primer sequences were then selected within the desired regions by screening for segments that are conserved across all oomycetes. Specificity of the primers was analysed by running BLASTn comparisons against the INSDc and manually by comparing against a custom ITS database containing sequences of nine major eukaryotic groups (Bengtsson-Palme et al. 2013).

PCR conditions and validation

PCR amplification was carried out using a reaction mixture consisting of 18 µl of PCR grade water, 5 µl of 5x HOT FIREPol Blend Mastermix (10 mM $MgCl_2$) (OÜ Solis Biodyne, Tartu, Estonia), 0.5 µl of both primers (20 µM) and 1 µl of DNA sample. Amplifications were done with Eppendorf 5341and Eppendorf 6321 thermal cyclers (Eppendorf AG, Hamburg, Germany) by running the following programme: 15 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C and a final cycle of 10 min at 72 °C.

The new forward primers were optimized for use with the universal reverse primer ITS4 (White et al. 1990) using OLIGOANALYZER 3.1 (https://eu.idtdna.com/calc/analyzer) to compare their calculated melting temperatures and GC content. The stability of possible homo- and heterodimers as well as hairpin structures was evaluated to avoid reduced amplification efficiency. All amplification tests were done at a 55 °C annealing temperature. Validation tests were carried out with DNA from various oomycete pure cultures as well as with DNA extracted from plants supposedly infected with pathogenic oomycetes and from 20 soil samples (Table 1). DNA from the pure cultures of five fungal species was used as negative control to test the specificity of the primers. The quality of all DNA samples used in specificity checks was tested by running PCR amplifications with universal ITS primers ITS1 and ITS4 (White et al. 1990).

Sequencing of infected plant samples

PCR products obtained from the six symptomatic plant samples were purified using to the ExoSAP method (Bell 2008) and Sanger-sequenced in Macrogen (Macrogen Europe, Amsterdam, The Netherlands). Sequencing was done with the oomycete-specific ITS1oo or ITS3oo primers as well as with the universal ITS4 primer. The obtained sequences were compared against the INSDc to confirm the identification.

High-throughput sequencing of soil samples

In total, 20 soil samples were sequenced using Illumina Miseq 2x300 PE HTS technology in the Estonian Biocentre (Tartu, Estonia). Amplicons were prepared with the primers ITS1oo and ITS4ngs (Tedersoo et al. 2014), both of which were tagged with one of the MID identifiers (cf. Tedersoo et al. 2014). The ITS1oo was used as the forward primer in order to sequence both ITS1 and ITS2 regions. PCR was performed as described above but in four replicates. PCR products were pooled and 5 µl of each product was resolved on 1% agarose gel to confirm amplification. Negative controls without template and positive controls containing DNA of *Aphanomyces astaci* were used in the sequencing process. The quantity of the products was normalized with the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA).

Analysis of Illumina sequencing data

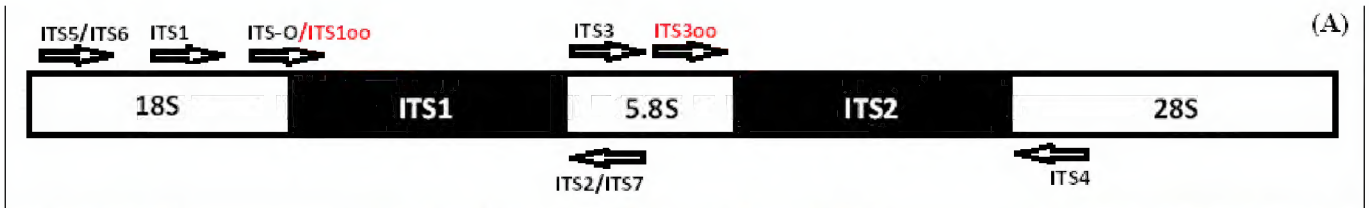
Based on sequencing primers, read 1 and read 2 were shuffled to contain regions of ITS1 and ITS2, respectively (using FQGREP (https://github.com/indraniel/fqgrep)). These paired-end reads were analysed separately, because in most cases the amplified full-length ITS region exceeded 600 bp and could not be merged. Sequencing reads were quality filtered and assigned to samples using MOTHUR (Schloss et al. 2009) (average quality over 15 bases ≥ 30). Potential chimeras were detected and removed using USEARCH 7.0.1090 (Edgar 2010). Sequences shorter than 150 bases were discarded and longer sequences were trimmed to 150 bases for clustering. The quality filtered ITS1 and ITS2 sequences were separately clustered to Operational Taxonomic Units (OTUs) based on 97% sequence similarity using CD-HIT (Li and Godzik 2006). The most abundant sequence was selected as a representative (using mothur) for BLASTn searches against a custom oomycete nucleotide database combined from the reference collections of Hyde et al. (2014) and Robideau et al. (2011) and INSDc. For each OTU, 10 best-matching references were determined for precise annotation. We considered OTUs to belong to oomycetes if they best matched known oomycetes. Oomycete OTUs with e-values < e^{-20} and identities above 80% were considered reliable enough to assign sequences to an order. OTUs with best matches other than oomycetes were assigned at the class level if e-value was < e^{-20} and identity above 75%.

Results

Primer selection and in silico analyses

As a result of aligning all oomycete ITS sequences present in the INSDc, it was possible to choose two short regions which are conserved across the majority of oomycetes and allow for the discrimination of other taxonomic groups. The primer ITS1oo overlaps with the primer ITS-O (Bachofer 2004) across 17 positions out of a total of 18 and is therefore not an original primer but a modification of ITS-O. This modification comes from a one bp shift which results in the deletion of a cytosine at the 5' end and the addition of an adenine at 3' end. The position of the added 3' adenine is polymorphic in other groups such as fungi and plants and should therefore make the modified ITS1oo more specific than the original ITS-O (Bachofer 2004). Primer coverage analysis of ITS1oo and ITS3oo shows that the primer sequences are conserved in nearly all known oomycete taxa. In case of ITS3oo, mismatches can be seen in some accessions of *Hyaloperonospora* and *Perofascia lepidii*. Both primers have significant mismatches in comparison to most other stramenopiles, fungi and plants (Figure 1).

The location of the 18 bp long ITS1oo, modified from the ITS-O (Bachofer 2004), covers 13 nucleotides at end of the ribosomal 18S gene and 5 nucleotides in the beginning of ITS1. The similarly 18 bp long ITS3oo is located at the end of the 5.8S gene, ending 7 nucleotides before the beginning of ITS2 (Figure 1). Both of the new primers were used as forward primers in combination with the universal reverse primer ITS4. The ITS4 was chosen due to its position at the beginning of the 28S gene, which allows for the amplification of both ITS1 and/or ITS2 when used together with ITS1oo or ITS3oo.



ITS1oo (5'-3') ITS-O (Bachofer 2004)	Mismatching taxon (% accessions)	ITS3oo (5'-3')	Mismatching taxon (% accessions) (B)
-GGAAGGATCATTACCACA		AGTATGYYTGTATCAGTG	
CGGAAGGATCATTACCAC-			
-	-	********M********	*Hyaloperonospora* (100%)
-	-	G*********G*****	*Perofascia* (100%)
WD****RNNNNN	*Fungi*	R*C*Y***Y**TYG**Y*	*Fungi*
**********RNNNNN	*Plantae*	G*C*C*****CC*GG*Y*	*Plantae*
**********Y*RH*	Other *Stramenopiles*	**C********CKG****	Other *Stramenopiles*
ITS6 (Cooke et al. 2000)		**ITS7 (Cooke et al. 2000)**	
GAAGGTGAAGTCGTAACAAGG		AGCGTTCTTCATCGATGTGC	
-	-	**********G*****	*Saprolegnia* (90%)
-	-	Y*Y*S**********	*Aphanomyces* (40%)
-	-	*****C**********	*Halophytophthora* (85%)
****TAA**********	*Fungi*	T**********CSA	*Fungi*
*****A**********	*Plantae*	TR**********CNA	*Plantae*
**********	Other *Stramenopiles*	T**********T****G	Other *Stramenopiles*

Figure 1. A Map of universal and oomycete-specific ITS region primers **B** Taxa with mismatches in the binding sites of primers ITS1oo and ITS3oo. Only taxa with 10% or more mismatching accessions are shown.

Analyses of pure culture and infected plant material

The primer pairs ITS1oo/ITS4 and ITS3oo/ITS4 produced a single amplification band of the expected length from all 15 tested oomycete strains, representing six genera (*Achlya*, *Aphanomyces*, *Phytophthora*, *Pythium*, *Saprolegnia*, *Scoliolegnia*) and eleven species. No visible bands were obtained in gel with DNA from five fungal species.

- Four samples, extracted directly from the symptomatic tissues of a grey alder (*Alnus incana*), a potato (*Solanum tuberosum*), a tomato (*Solanum lycopersicum*) and a goutweed (*Aegopodium podagraria*), produced a single amplification band with both primer pairs ITS1oo/ITS4 and ITS3oo/ITS4 and were sequenced. Sequencing of the grey alder sample was successful with the primer ITS3oo, whereas the other three samples were successfully sequenced with both ITS1oo and ITS3oo. Comparisons against the NCBI GenBank nucleotide database showed that the sequence from the first sample belongs to *Phytophthora* sp. (99% similarity), the sequences from the second and third samples belong to *Phytophthora infestans* (100% and 99% similarity) and the sequence from the goutweed sample belongs to *Plasmopara nivea* (99% similarity). One sample from a zucchini plant (*Cucurbita pepo*) and one from a grape vine (*Vitis vinifera*) produced multiple amplification bands of different sizes with both primer pairs and were not sequenced.

Soil sample oomycete diversity

Altogether 67133 quality filtered ITS1 reads were recovered from the 20 soil samples. In all, 281 singletons were discarded from further analyses. Nearly 66% of all reads belonged to unknown taxa, 25% to oomycetes and 9% to other taxonomic groups (Figure 2). The quality filtered ITS1 sequences were clustered into 1820 OTUs based on 97% similarity threshold, 30% of which were assigned to a known class or order. Out of the 554 assigned OTUs, nearly 73% belonged to oomycetes, 16% to fungi and 9% to plants. Of 404 oomycete OTUs, 307 were assigned to a known order. On average, oomycetes comprised 61 OTUs (range, 13-94) represented by 32% (range, 1-66%) of reads in soil samples (Figure 3).

For the ITS2 subregion, 77734 quality filtered reads comprised 1720 OTUs and 241 singletons. Out of all ITS2 reads, 30% were assigned to oomycetes and 8% to fungi, whereas 60% belonged to unknown taxa (Figure 2). Oomycetes comprised 493 of the 672 identified taxa (73%). In total, 333 of these taxa were assigned to a known order. The number of oomycete OTUs averaged 86 (range, 42–148) per soil sample (Figure 3). On average, oomycetes contributed to 36% (range, 12–69%) in soil samples.

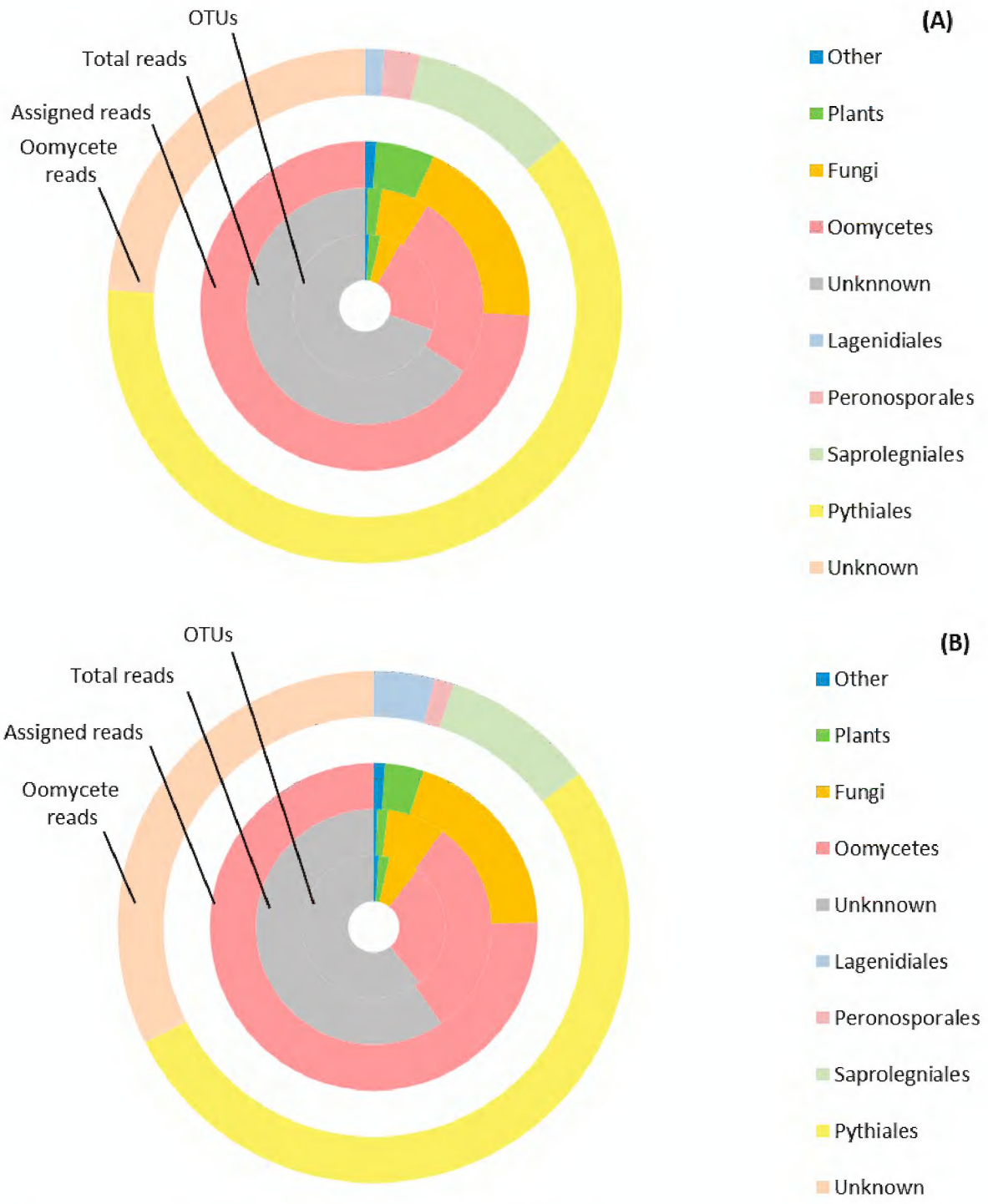


Figure 2. OTU and read distributions of ITS1 (**A**) and ITS2 (**B**) reads. Panels starting from outermost: **1** Oomycete read distribution between orders **2** Read distribution between classes, excluding reads of unknown origin **3** Read distribution between classes, including reads of unknown origin **4** OTU distribution between classes.

Discussion

The ultimate aim of this study was to validate an alternative method for metabarcoding oomycetes in complex substrates such as soil. We developed a novel taxon-specific PCR assay for the ITS region-based identification of oomycetes. When compared with the previously developed ITS-O, ITS6 and ITS7 primers, the ITS1oo, modified from the original ITS-O (Bachofer 2004), and the newly designed ITS3oo exhibit somewhat greater *in silico* specificity for oomycetes. In comparison to the ITS-O, the modified ITS1oo includes an additional 3' terminal adenine, a position that is polymorphic in fungi and plants and should therefor add to the specificity of the primer. Based on our

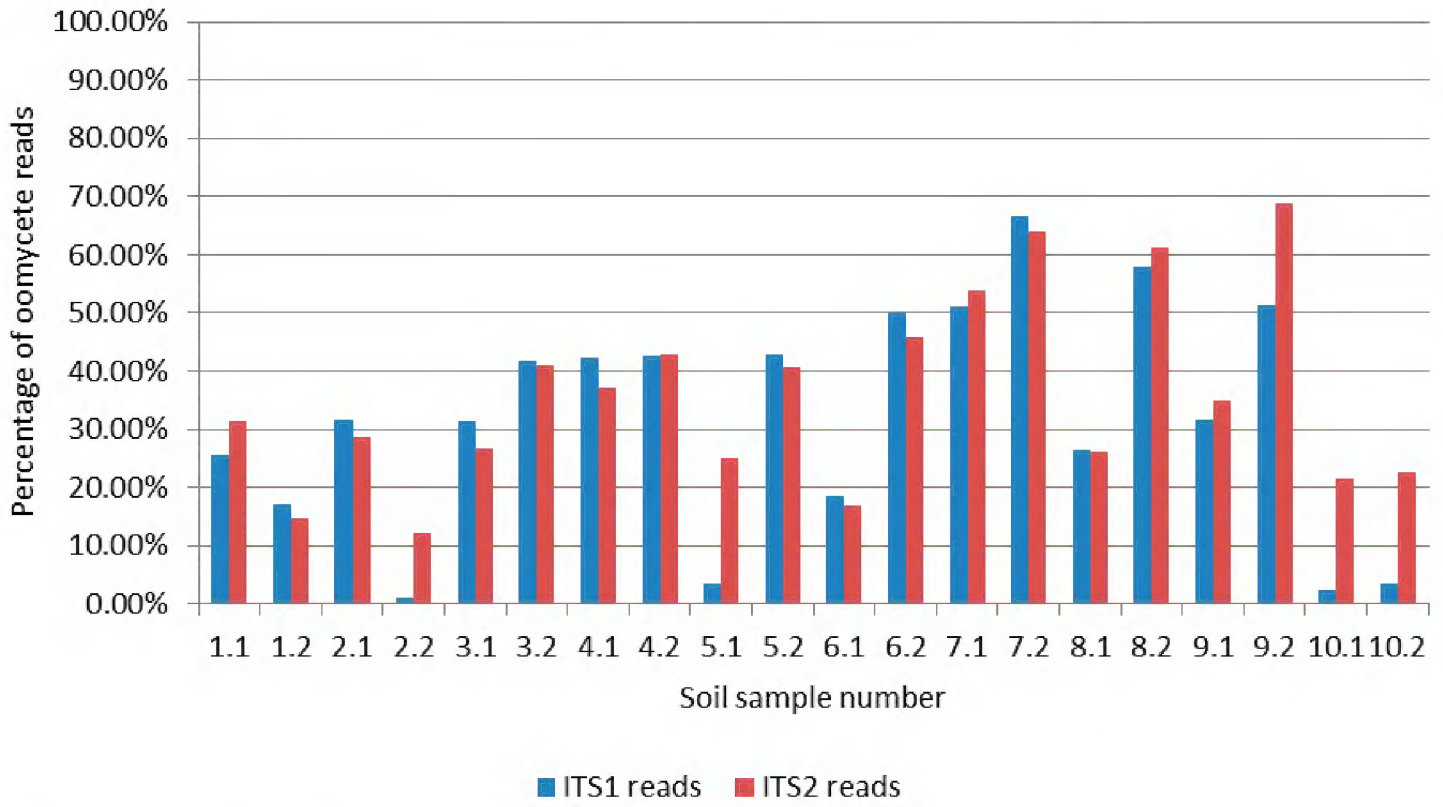


Figure 3. Fraction of oomycete reads in individual soil samples.

analyses and in contrast to Sapkota and Nicolaisen (2015), the primer ITS6 has only one mismatching position in comparison to the majority of corresponding plant accessions in the INSDc, whereas the ITS1oo has several mismatches in the 3′ end. This may significantly lower the specificity of the ITS6, as a single internal mismatch does not reduce the amplification efficiency markedly (Kwok et al. 1990). In addition, the ITS6 has no mismatches compared to the majority of non-oomycete stramenopile accessions, while the ITS1oo has 3′ mismatches against several non-oomycete stramenopile groups. Both ITS1oo and ITS6 show complete coverage of all oomycete groups present in the INSDc, whereas the ITS7 has one mismatch in comparison to the accessions of *Saprolegnia* (Sapkota and Nicolaisen 2015) and *Halophytophthora* and 2-3 mismatches against four species of the known pathogenic genus *Aphanomyces* (Sapkota and Nicolaisen 2015). The presence of two or more mismatches can limit the usability of ITS7 in detecting these taxa, especially when using relatively high annealing temperatures (Sipos et al. 2007) as suggested by Sapkota and Nicolaisen (2015). In comparison, the primer ITS3oo has a single mismatch compared to the accessions of genus *Hyaloperonospora* and two mismatches against the single known species of *Perofascia*.

Furthermore, the modified and newly developed forward primers are located in the very end of the conserved fragments that reduce the size of amplicons by 10-20% compared with the ITS6 forward primer, which is of great importance for HTS platforms producing short fragments such as Illumina and Ion Torrent. When combined with universal reverse primers, these oomycete-specific primers could be used in multiplex with other specific forward primers to address several taxonomic groups of pathogens simultaneously, without adding the cost of multiple barcoded reverse primers (Tedersoo et al. 2015).

Previous studies have used oomycete-specific primers ITS6 and ITS7 to amplify the ITS1 region with highly variable success. For example, Vannini et al. (2013) recovered only 23 oomycete OTUs from 10 forest soil samples, where oomycetes contributed to 79% of all reads. More recently, Coince et al. (2013) recovered a total of 10 oomycete OTUs from 20 samples of forest soil that contributed to 15% of all reads. Sapkota and Nicolaisen (2015) improved the ITS6/ITS7 based method by optimizing the annealing temperature and as a result recovered 67 oomycete OTUs (95% of all reads) from 26 agricultural soil samples, but may have missed multiple taxa due to overly strict PCR conditions. Furthermore, it should be noted that fine tuning of PCR conditions is only possible in-house, because PCR buffer including salts ($MgCl_2$) and stabilizers (BSA), the type of polymerase and concentration of primers and templates all affect primer specificity (Innis et al. 1990, Cha and Thilly 1993).

In this study, we recovered 404 ITS1-based and 493 ITS2-based oomycete OTUs from 20 soil samples from forest nurseries and bordering control areas. The number of recovered oomycete OTUs is considerably higher than in previous studies, which could be due to higher diversity in the analysed soil samples or a result of some properties of the new assay. Oomycete reads comprised on average 32% and 36% of the total reads of individual soil samples for ITS1 and ITS2, respectively. The assigned oomycete OTUs belonged to the orders of *Lagenidiales*, *Peronosporales*, *Pythiales* and *Saprolegniales*, confirming the ability of the proposed new assay to detect various oomycete groups from complex samples. *Pythiales* were found to be dominating in the soil samples, making up nearly 50% of the total oomycete reads, a result that is in line with previous oomycete community studies (Arcate et al. 2006, Sapkota and Nicolaisen 2015).

The new primers were also used to identify oomycete pathogens from infected plant samples by using Sanger sequencing. The pathogens were successfully determined in four samples out of six. Sequencing was successful with both ITS1oo and ITS3oo from a goutweed (*Aegopodium podagraria*), a potato (*Solanum tuberosum*) and a tomato (*Solanum lycopersicum*) sample, whereas in the case of a grey alder (*Alnus* incana) sample only ITS3oo produced an identifiable sequence. This could indicate a somewhat higher specificity of ITS3oo in comparison to ITS1oo in some cases when identifying pathogens from infected plant material. Two samples out of six produced multiple amplification bands, possibly indicating the presence of several oomycete species in the infected sample. This result shows that the new primers can be used to detects oomycete pathogen species directly from infected plant samples in cases where the infected tissue in dominated by one pathogen, without co-amplification of plant and fungal DNA.

Taken together, we provide highly oomycete-specific forward primers that can be used in combination with previously developed oomycete-specific or universal reverse primers. Considering the rapid evolution of high-throughput sequencing, the full ITS sequence is certainly preferable over ITS1 or ITS2 used alone, because these subregions may differ in the taxonomic resolution across genera.

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References

Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V, Polz MF (2005) PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Applied and Environmental Microbiology 71: 8966–8969. doi: 10.1128/AEM.71.12.8966-8969.2005

Arcate JM, Karp MA, Nelson EB (2006) Diversity of Peronosporomycete (oomycete) communities associated with the rhizosphere of different plant species. Microbial Ecology 51: 36–50. doi: 10.1007/s00248-005-0187-y

Bachofer M (2004) Molekularbiologische Populationsstudien an Plasmopara halstedii, dem Falschen Mehltau der Sonnenblume Dissertation, Universitat Hohenheim Germany, 1–140.

Bell J (2008) A simple way to treat PCR products prior to sequencing using ExoSAP-IT. Biotechniques 44: 834. doi: 10.2144/000112890

Bengtsson-Palme J, Ryberg M, Hartmann M, Branco S, Wang Z, Godhe A, De Wit P, Sánchez-García M, Ebersberger I, de Sousa F, Amend A, Jumpponen A, Unterseher M, Kristiansson E, Abarenkov K, Bertrand YJK, Sanli K, Eriksson KM, Vik U, Veldre V, Nilsson RH (2013) Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods in Ecology and Evolution 4: 914–919. doi: 10.1111/2041-210X.12073

Cha RS, Thilly WG (1993) Specificity, Efficiency, and Fidelity of PCR. Genome Research 3: S18–S29. http://genome.cshlp.org/content/3/3/S18.long

Choi Y-J, Beakes G, Glockling S, Kruse J, Nam B, Nigrelli L, Ploch S, Shin H-D, Shivas RG, Telle S, Voglmayr H, Thines M (2015) Towards a universal barcode of oomycetes – a comparison of the *cox*1 and *cox*2 loci. Molecular Ecology Resources 15: 1275–1288. doi: 10.1111/1755-0998.12398

Coince A, Cael O, Bach C, Lengelle J, Cruaud C, Gavory F, Morin E, Murat C, Marcais B, Buee M (2013) Below-ground fine-scale distribution and soil versus fine root detection of fungal and soil oomycete communities in a French beech forest. Fungal Ecology 6: 223–235. doi: 10.1016/j.funeco.2013.01.002

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM (2000) A molecular phylogeny of Phytophthora and related oomycetes. Fungal Genetics and Biology 30: 17–32. doi: 10.1006/fgbi.2000.1202

Dick MW (2001) Straminipilous fungi. Kluwer Academic Publishers, 1–670. doi: 10.1007/978-94-015-9733-3

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19): 2460-1. doi: 10.1093/bioinformatics/btq461

Herrero ML, Toppe B, Brurberg MB (2011) First report of Phytophthora ramorum causing shoot dieback on bilberry (Vaccinium myrtillus) in Norway. Plant Disease 95(3): 355. doi: 10.1094/PDIS-10-10-0709

Hudspeth DSS, Nadler SA, Hudspeth MES (2000) A cox2 phylogeny of the Peronosporomycetes (Oomycetes). Mycologia 92: 674–684. doi: 10.2307/3761425

Hyde KD, Henrik Nilsson R, Aisyah Alias S, Ariyawansa HA, Blair JE, Cai L, de Cock WAMA, Dissanayake AJ, Glockling SL, Goonasekara ID, Gorczak M, Hahn M, Jayawardena RS, van Kan JAL, Laurence MH, André Lévesque C, Li X, Liu J-K, Maharachchikumbura SSN, Manamgoda DS, Martin FN, McKenzie EHC, McTaggart AR, Mortimer PE, Nair PVR, Pawłowska J, Rintoul TL, Shivas RG, Spies CFJ, Summerell BA, Taylor PWJ, Terhem RB, Udayanga D, Vaghefi N, Walther G, Wilk M, Wrzosek M, Xu J-C, Yan JY, Zhou N (2014) One stop shop: backbones trees for important phytopathogenic genera: I. Fungal Diversity 67: 21–125. doi: 10.1007/s13225-014-0298-1

Innis MA, Gelfand DH, Sninsky JJ, White TJ (1990) PCR Protocols A Guide to Methods and Applications. Academic Press, 1–482. doi: 10.1016/0167-7799(90)90215-J

Ishii K, Fukui M (2001) Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. Applied and Environmental Microbiology 67(8): 3753–5. doi: 10.1128/AEM.67.8.3753-3755.2001

Kamoun S (2003) Molecular genetics of pathogenic oomycetes. Eukaryotic Cell 2: 191–199. doi: 10.1128/EC.2.2.191-199.2003

Karling JS (1981) Predominantly holocarpic and eucarpic simplebiflagellate phycomycetes. J Cramer, Vaduz, 1–252.

Kwok S, Kellogg DE, McKinney N, Spasic D, Godal L, Levenson C, Sninsky JJ (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Research 18: 999–1005. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC330356/pdf/nar00188-0292.pdf

Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22(13): 1658–9. doi: 10.1093/bioinformatics/btl158

Robideau GP, De Cock AW, Coffey MD, Voglmayr H, Brouwer H, Bala K, Chitty DW, Désaulniers N, Eggertson QA, Gachon CM, Hu CH, Küpper FC, Rintoul TL, Sarhan E, Verstappen EC, Zhang Y, Bonants PJ, Ristaino JB, Lévesque CA (2011) DNA barcoding of oomycetes with cytochrome *c* oxidase subunit I and internal transcribed spacer. Molecular Ecology Resources 11(6): 1002–1011. doi: 10.1111/j.1755-0998.2011.03041.x

Sapkota R, Nicolaisen M (2015) An improved high throughput sequencing method for studying oomycete communities. Journal of Microbiological Methods 110: 33–39. doi: 10.1016/j.mimet.2015.01.013

Sipos R, Székely AJ, Palatinszky M, Révész S, Márialigeti K, Nikolausz M (2007) Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. FEMS Microbiology Ecology 60: 341–350. doi: 10.1111/j.1574-6941.2007.00283.x

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75(23): 7537–41. doi: 10.1128/AEM.01541-09

Schmidt P-A, Bálint M, Greshake B, Bandow C, Römbke J, Schmitt I (2013) Illumina metabarcoding of a soil fungal community. Soil Biology and Biochemistry 65: 128–132. doi: 10.1016/j.soilbio.2013.05.014

Sparrow FK (1960) Aquatic Phycomycetes, 2nd revised edition. University of Michigan Press, Ann Arbor, 1–1187. https://archive.org/stream/aquaticphycomyce00spar/aquaticphycomyce00spar_djvu.txt

Sparrow FK (1976) The present status of classification in biflagellate fungi. In: Gareth-Jones EB (Eds) Recent advances in aquatic mycology. Elek Science, London, 213–222. doi: 10.1002/jobm.19790190115

Spring O, Bachofer M, Thines M, Riethmüller A, Göker M, Oberwinkler F (2006) European Journal of Plant Pathology 114: 309. doi: 10.1007/s10658-005-5996-9

Tedersoo L, Bahram M, Cajthaml T, Põlme S, Hiiesalu I, Anslan S, Harend H, Buegger F, Pritsch K, Koricheva J, Abarenkov K (2016) Tree diversity and species identity effects on soil fungi, protists and animals are context-dependent. ISME Journal 10: 346–362. doi: 10.1038/ismej.2015.116

Tedersoo L, Bahram M, Põlme S, Kõljalg U, Yorou NS, Wijesundera R, Villarreal Ruiz L, Vasco-Palacios AM, Thu PQ, Suija A, Smith ME, Sharp C, Saluveer E, Saitta A, Rosas M, Riit T, Ratkowsky D, Pritsch K, Põldmaa K, Piepenbring M, Phosri C, Peterson M, Parts K, Pärtel K, Otsing E, Nouhra E, Njouonkou AL, Nilsson RH, Morgado LN, Mayor J, May TW, Majuakim L, Lodge DJ, Lee SS, Larsson KH, Kohout P, Hosaka K, Hiiesalu I, Henkel TW, Harend H, Guo LD, Greslebin A, Grelet G, Geml J, Gates G, Dunstan W, Dunk C, Drenkhan R, Dearnaley J, De Kesel A, Dang T, Chen X, Buegger F, Brearley FQ, Bonito G, Anslan S, Abell S, Abarenkov K (2014) Global diversity and geography of soil fungi. Science 346(6213): 1078. doi: 10.1126/science.1256688

Thines M (2007) Characterisation and phylogeny of repeated elements giving rise to exceptional length of ITS2 in several downy mildew genera (Peronosporaceae). Fungal Genetics and Biology 44(3): 199–207. doi: 10.1016/j.fgb.2006.08.002

Vannini A, Bruni N, Tomassini A, Franceschini S, Vettraino AM (2013) Pyrosequencing of environmental soil samples reveals biodiversity of the Phytophthora resident community in chestnut forests. FEMS Microbiology Ecology 85(3): 433–42. doi: 10.1111/1574-6941.12132

Vettraino AM, Bonants P, Tomassini A, Bruni N, Vannini A (2012) Pyrosequencing as a tool for the detection of Phytophthora species: error rate and risk of false Molecular Operational Taxonomic Units. Letters in Applied Microbiology 55(5): 390–6. doi: 10.1111/j.1472-765x.2012.03310.x

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds) PCR protocols: a guide to methods and applications. Academic Press, New York, 315–322. doi: 10.1016/B978-0-12-372180-8.50042-1